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Sulfur Mustard Induces Apoptosis in Cultured Normal Human Airway Epithelial Cells: Evidence of a Dominant Caspase-8-mediated Pathway and Differential Cellular Responses

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We have shown that sulfur mustard (SM; bis-(2-chloroethyl) sulfide), an alkylating, vesicating chemical warfare agent, causes dermal toxicity, including skin microblisters, via the induction of both death receptor (DR) and mitochondrial pathways of apoptosis in human epidermal keratinocytes. While SM is known for its skin-vesicating properties, respiratory tract lesions are the main source of morbidity and mortality after inhalation exposure. We, therefore, investigated whether SM induces apoptotic cell death in normal human bronchial epithelial (NHBE) cells and small airway epithelial cells (SAEC) *in vitro*. Cells were exposed to various concentrations of SM (0, 50, 100, and 300 μM for 16 h) in the culture medium and then tested for the activation of apoptotic executioner caspase-3 and initiator caspases-8 and -9. Caspases-8 and -3 were activated by SM in both airway cell types, indicating the induction of a DR pathway of

apoptosis in these cells; however, the levels of enzyme activation were different, depending on the cell type and the SM concentrations used. Consistent with enzyme activity results, immunoblot analyses revealed the proteolytic processing of the proenzymes to the active forms of caspases-8 and -3 in these cells after SM exposure. Interestingly, NHBE cells were found to be exquisitely sensitive to SM, compared to SAEC, with caspase-3 activities in SM-exposed NHBE cells ~2-fold higher and caspase-8 activities ~10-fold higher than in SAEC. Furthermore, SM activated caspase-9 in NHBE cells, but not in SAEC, indicating a possible role of the mitochondrial pathway only in the NHBE cells. The present study shows that both upper airway (NHBE cells) and deep lung (SAEC) epithelial cells undergo SM-induced apoptotic death *in vitro*, but distinct cell-type specific responses can be elicited, which may be attributed to intrinsic properties that characterize the response of these cells to SM. These findings need to be taken into consideration in the search for modulators of these pathways for the therapeutic intervention to reduce SM injury due to respiratory tract lesions.

INTRODUCTION

Sulfur mustard (SM; bis-(2-chloroethyl) sulfide), the highly reactive vesicant agent used for chemical warfare in World War I and the Iraq/Iran conflict, is now reemerging as a major threat not only to troops, but also to civilians (Saladi et al., 2006). SM causes severe skin blisters by inducing death and detachment of the basal cells of the epidermis (Meier et al., 1984; Gross et al., 1988; Petrali et al., 1990; Smith et al., 1990; Papirmeister et al., 1991; Smith et al., 1991). Although SM is known for its ability to cause severe skin blistering, most mortality associated with SM is attributed to pulmonary damage and respiratory tract lesions (Urbannetti, 1988). Evidence of epithelial damage to the skin, eyes, and respiratory tract has been reported (Papirmeister et al., 1991; Etezad-Razavi et al., 2006; Balali-Mood and Hefazi, 2006; Dacre and Goldman, 1996). Respiratory complications of SM poisoning in Iranian veterans included laryngitis, tracheobronchitis, bronchiolitis, bronchopneumonia, chronic obstructive pulmonary disease (COPD), bronchiectasis, asthma, large airway narrowing, and pulmonary fibrosis (Balali-Mood and Hefazi, 2006; Ghanei and Harandi, 2007; Bijani and Moghadamnia, 2002; Hefazi et al., 2005; Emad and Emad, 2007). The mechanisms that underlie these SM-induced respiratory lesions, however, remain to be elucidated.

We have shown that while human dermal fibroblasts contribute to the vesication response in SM-exposed skin by releasing degradative cytosolic components after a PARP (poly(ADP-ribose)polymerase)-dependent necrosis caused by SM (Rosenthal et al., 2001), SM toxicity in normal human epidermal keratinocytes (NHEKs) is primarily due to the induction of both terminal differentiation and apoptosis (Rosenthal et al., 1998). Two main signaling

pathways have been proposed to contribute to SM-induced apoptosis in NHEK. These are (i) an extrinsic DR-mediated pathway and (ii) an intrinsic mitochondrial pathway. The two pathways are not necessarily mutually exclusive and cross-talk may occur between them as a mechanism of amplification. The DR pathway is initiated by the clustering and activation of the membrane receptors (Fas), leading to the formation of a death-inducing signaling complex (DISC). The DISC contains an adaptor protein consisting of FADD (Fas-associated death domain protein), which contains a DD (death domain) and a DED (death effector domain) that recruits and leads to autocatalytic activation of the initiator procaspase-8. Caspase-8 then cleaves and activates the effectors, caspases-3 and -7. The mitochondrial pathway occurs primarily, although not solely, as the result of DNA damage signaling to the mitochondria. The effects are the release of cytochrome C and other apoptogenic factors (e.g., Smac/Diablo) that, together with Apaf-1 and ATP/dATP, trigger the formation of the apoptosome and procaspase-9 activation. Caspase-9 then cleaves and activates the effectors, caspases-3 and -7, that orchestrate downstream apoptotic events. The "commitment" to the release of proapoptotic factors from the mitochondria depends primarily on the balance between pro- and antiapoptotic members of the Bcl2 family of proteins. Bcl2 and BclxL stabilize mitochondrial integrity, while Bax and Bak destabilize this organelle. When Bax or Bak are induced or activated, they insert into the mitochondrial outer membrane, causing cytochrome C release and apoptosis. Conversely, Bcl2 overexpression blocks the effects of DNA damage-induced Bax activation, cytochrome C release, and, thus, apoptosis.

We have reported that NHEK apoptosis due to SM exposure can be inhibited by suppressing either the mitochondrial response (calmodulin (CaM) → Bcl2 → caspase-9 activation) via the expression of CaM-antisense (Simbulan-Rosenthal et al., 2006) or the DR response (Fas → FADD → caspase-8 activation) via the expression of dominant-negative FADD (DN-FADD) constructs (Rosenthal et al., 2003). These observations led to our conclusion that SM induces apoptosis in keratinocytes *via* both mitochondrial and DR pathways (Rosenthal et al., 2000; Rosenthal et al., 2003, Simbulan-Rosenthal et al., 2006).

In the present study, we investigated the effects of SM exposure on the induction of apoptosis pathways in NHBE cells (large airway) and SAEC (small airway, deep lung) *in vitro*. Our goal was to see whether the pathways could be modulated to alter SM toxicity in cell culture and possibly in an animal model in subsequent studies. Results presented here indicate that SM induces apoptosis in NHBE cells and SAEC through a DR-mediated pathway and that there are distinct responses in different locations of the respiratory tract (from upper to lower, large versus small airways). These findings on the mechanisms for SM-induced pulmonary damage could lead to therapeutic strategies for the prevention or treatment of SM toxicity.

MATERIALS AND METHODS

Chemicals and Cells

SM (*bis*-(2-chloroethyl) sulfide; >98% purity) was obtained from the US Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, Maryland, USA). Frozen stock NHBE cells, and SAECs) as well as their growth media (BEGM, SAGM) were obtained from Lonza (Walkersville, Maryland, USA). NHBE cells and SAECs were maintained in serum-free growth media, BEGM or SAGM, respectively, supplemented with bovine pituitary extract, hEGF, hydrocortisone, epinephrine, transferrin, insulin, and retinoic acid. Since these cells become irreversibly contact inihibited, they were subcultured or used before they reached an 80% confluence and up to the third passage.

For exposure to SM, cells were grown to ~80%–90% confluency (visual approximation) and then exposed to SM diluted in BEGM or SAGM to final concentrations of 50, 100, or 300 µM. SM undergoes a rapid hydrolysis in aqueous solution and was, therefore, diluted in cell growth medium immediately before use. Cells were exposed to diluted SM in growth medium, as described previously (Ray et al., 1995). Briefly, 10 µL of neat SM placed under 10 mL of frozen cell growth medium was melted by warming to room temperature, and vortexed at top speed for 1 min to solubilize SM in the medium and produced a 4-mM stock solution. The stock solution was then added to the cell culture medium in flasks to achieve the desired SM concentrations, and this medium was not changed for the duration of the experiments. At indicated time points after SM exposure, cells were harvested for further analyses.

Fluorometric Assay of Caspase Activity

Cytosolic extracts were prepared from total cells (pooled floating and attached cells) grown in 150 cm^2 flasks and used in fluorometric assays of caspase activity. Caspase-3 activity assays were based on hydrolysis of the fluorogenic tetrapeptide substrate specific for caspases-3 Ac-DEVD-AMC ([N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin]; BioMol, Conshohocken, PA, USA), as previously described (Simbulan-Rosenthal et al., 2002). For the fluorometric caspase-8 and -9 activity assays, the tetrapeptide substrates specific for caspases-8 and -9 (Ac-IETD [Ile-Glu-Thr-Asp]-AMC and Ac-LEHD [Leu-Glu-His-Asp]-AMC, respectively; BioMol, Conshohocken, PA, USA) were utilized under the same conditions as caspase-3. For comparison of results, the same amount of protein (25 µg in cytosolic extracts) was used in all assays. Caspases are cysteine proteases that cleave their substrates after aspartic acid residues (cysteine aspartase). Thus, in these assays, free fluorescent AMC, generated as a result of the cleavage of the aspartate-AMC bond, was monitored over 30 min with a Wallac Victor[3]V fluorometer (Perkin Elmer; Shelton, Connecticut, USA) at excitation and emission wavelengths of 360 and

460 nm, respectively. The emission from each sample was plotted against time, and linear regression analysis of the initial velocity (slope) for each curve yielded the activity.

Immunoblot Analysis

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transfer of separated proteins to nitrocellulose membranes were performed according to standard protocols (Laemmli, 1970). Total protein content was measured (DC protein assay; BioRad, Hercules, CA, USA), and Ponceau S (0.1%) (Sigma-Aldrich, Saint Louis, MO, USA) staining (Nakamura et al., 1985) of membranes was performed to verify equal loading and transfer of proteins. Membranes were then incubated with antibodies to the p17 subunit of caspase-3 (1:200; Santa Cruz Biotech, Santa Cruz, CA, USA), procaspase-8 (1:1000; PharMingen, San Diego, CA, USA), and caspase-9 (1:500; Calbiochem, San Diego, CA, USA). Immune complexes were detected by subsequent incubation with appropriate horseradish peroxidase–conjugated antibodies to mouse or rabbit IgG (1:3000) and enhanced chemiluminescence (Pierce, Rockford, IL, USA). Immunoblots were sequentially stripped of antibodies by incubation for 30 min at 50°C with a solution containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7), blocked again, and reprobed with additional antibodies to compare different proteins from the same filter (Rosenthal et al., 2003). Typically, a filter could be reprobed three times before there was a detectable loss of protein from the membrane, which was monitored by Ponceau S staining after stripping.

RESULTS

SM Induces Apoptosis in SAEC as Shown by Activation of Caspases-3 and -8

To delineate the mechanisms that underlie SM-induced respiratory lesions, we investigated whether SM exposure of SAEC induces apoptotic responses similar to those that have been observed for NHEK. Since caspase-3 has been shown to be a point of convergence for different apoptotic pathways, we first focused on caspase-3 activation in the SM apoptotic response. We also assayed for the activation of other key caspases, in particular the upstream initiator caspase-8, which is activated in the DR pathway of apoptosis. Quantitative fluorometric caspase-3 and -8 activity assays on cytosolic extracts prepared from SAEC exposed to increasing concentrations of SM revealed a significant increase in both caspase-3 and -8 activities at 16 h after exposure (Fig. 1A, left panels). The extent of caspase-3 stimulation was dependent on SM concentration, the highest concentration used being 300 μM, which is

A

B
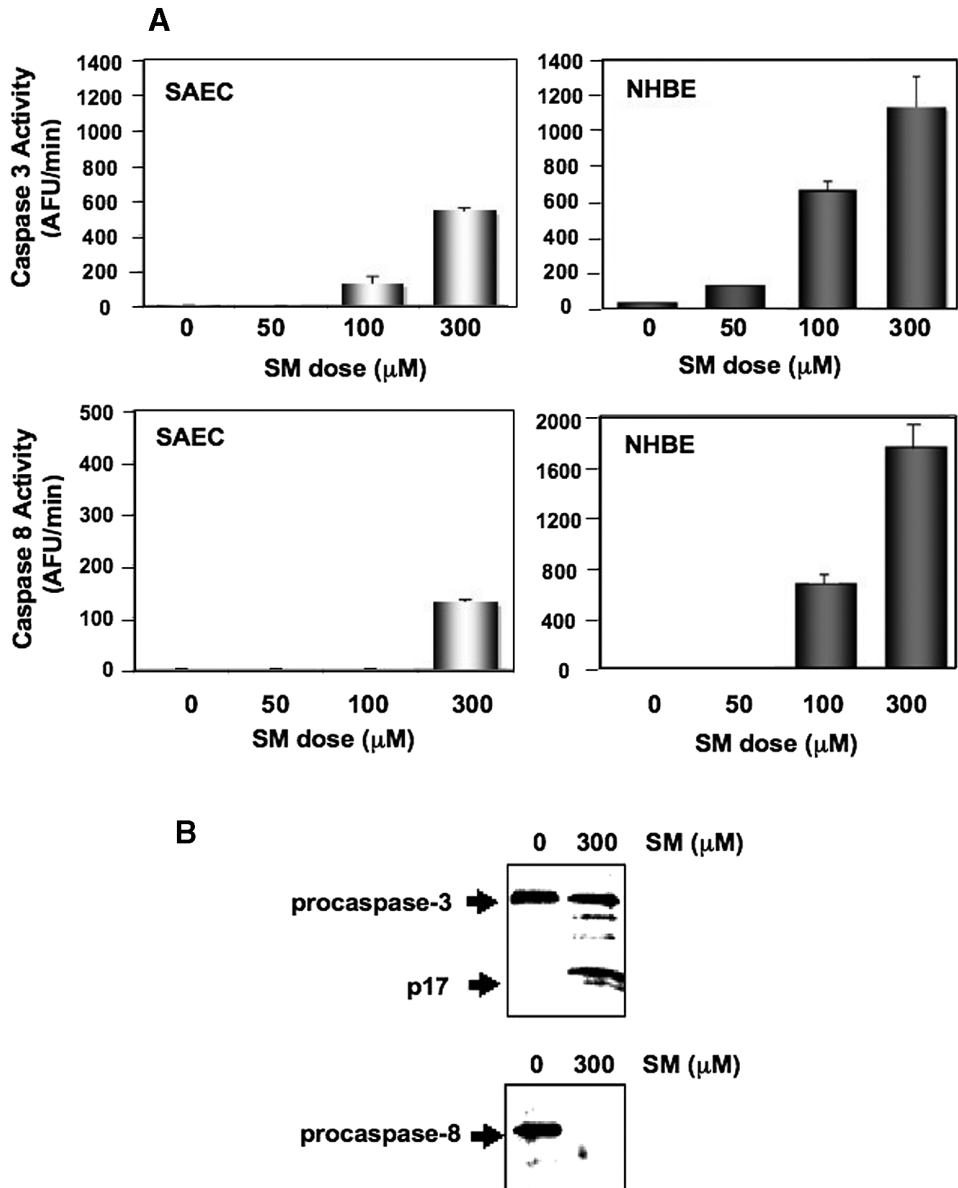
Figure 1: Sulfur mustard (SM)-induced activation of caspases-3 and -8 in normal human small airway epithelial cells (SAECs) compared to normal human bronchial epithelial (NHBE) cells. (A) SAEC and NHBE cells were exposed to the indicated concentrations of SM, and cytosolic extracts were prepared after 16 h and subjected to quantitative fluorometric caspase-3 activity assays with Ac-DEVD-AMC (for caspase-3) or Ac-IETD-AMC (for caspase-8) as substrates. Data are presented as the mean ± standard deviation of three replicates of a representative experiment; essentially, the same results were obtained in three independent experiments. (B) SAEC cytosolic extracts that were prepared after 16 h in (A) were also subjected to immunoblot analysis, using antibodies specific for executioner caspase-3 (upper panel) and initiator capsase-8 (lower panel). The positions of the immunoreactive proteins are indicated.

considered to be the *in vitro* equivalent of a vesicating dose *in vivo* (William J. Smith, USAMRICD, APG, MD, personal communication). Caspase-8 activity was also induced after exposure to 300 µM SM. Consistent with the results of caspase activity assays and similar to keratinocyte responses to SM (Rosenthal et al., 2003), complete proteolytic processing of a significant fraction of procaspase-3 (p32) to the active form (p17) was noted in SAEC after exposure to 300 µM SM (Fig. 1B). Reprobing of the immunoblot with antibodies to the initiator caspase-8 also revealed SM-induced proteolytic processing of caspase-8, as shown by the disappearance of the intact zymogen form in SM-exposed SAEC.

NHBE Cells are More Sensitive to SM than SAEC as Shown by Activation of Caspases-3 and -8 at Lower SM Concentrations

We next investigated the effects of SM exposure on NHBE cells compared to SAEC. NHBE cells and SAEC were exposed to increasing concentrations of SM and 16 h after exposure, cytosolic extracts were tested for the activation of caspases-3 and -8. In both cell types, SM stimulated apoptotic caspases-3 and -8; the extent of stimulation was dependent on the SM concentrations used (Fig. 1A). Large airway NHBE cells exhibited increased sensitivity to SM compared to small airway SAEC, as evidenced by a ~2-fold higher caspase-3 activity (Fig. 1A, upper panels) and a ~10-fold higher caspase-8 activity (Fig. 1A, lower panels) in response to 300 µM SM in NHBE cells. Moreover, the NHBE cells, but not SAEC, showed activation of caspase-3 and -8 at lower SM concentrations (50 and 100 µM). Taken together, these results suggest that both NHBE cells and SAEC undergo a DR- and caspase-8-mediated apoptotic pathway upon SM exposure, and that large airway epithelial cells exhibit an increased sensitivity to SM, compared to the small airway cells.

SM Activates Caspase-9 in NHBE Cells, but Not in SAEC

We next investigated the possible reasons for the differential sensitivity of NHBE cells and SAEC to SM exposure and the potential involvement of the mitochondrial pathway of apoptosis in these responses. NHBE cells and SAEC were exposed to increasing concentrations of SM; cytosolic extracts were prepared after 16 h and subjected to fluorometric caspase activity assays for caspase-9, using Ac-LEHD-AMC as a substrate. SAECs did not show any notable activation of caspase-9, even at 300 µM SM (vesicating dose). In contrast, the large airway NHBE cells exhibited a SM concentration–dependent activation of caspase-9, beginning at 100 µM and activity increased ~5-fold at 300 µM (Fig. 2A). Consistent with the results of the caspase activity assays, immunoblot analyses revealed that, while 300 µM SM induced proteolytic processing of procaspase-3 into its active p17 form in both SAEC and NHBE cells,

A

B
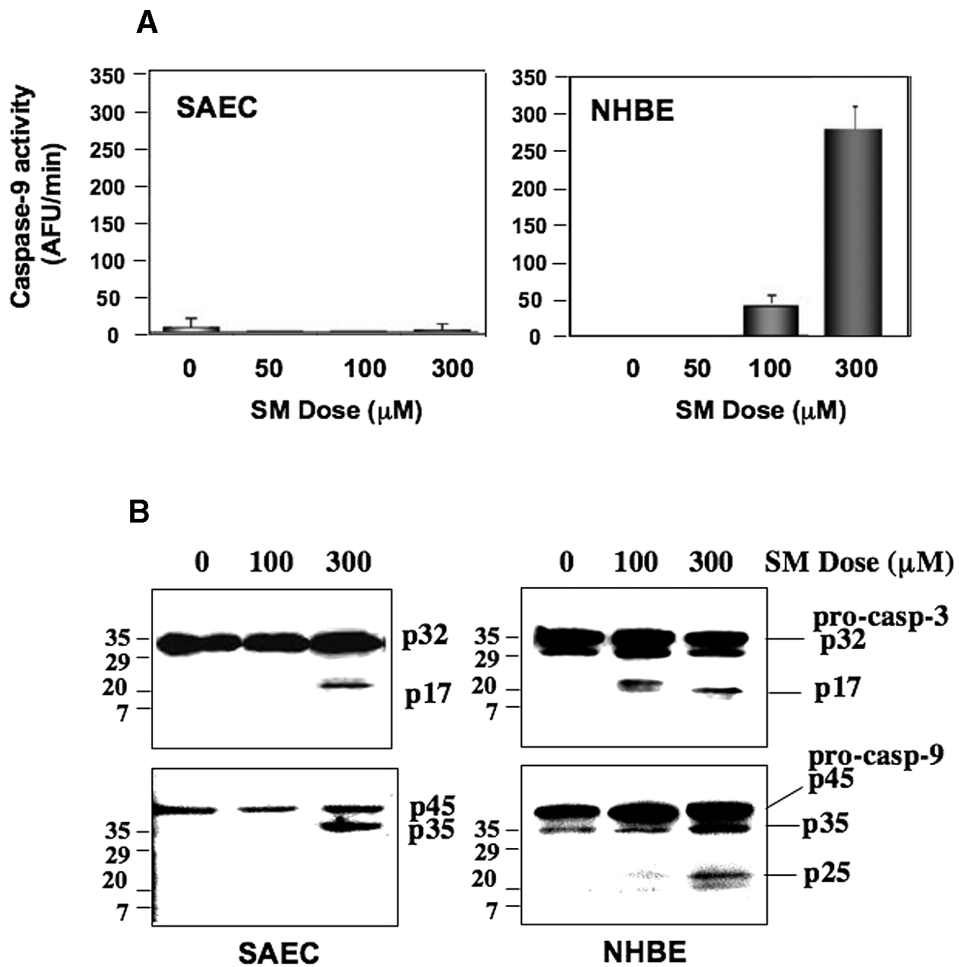
Figure 2: Sulfur mustard (SM)-induced activation of caspase-9 in normal human bronchial/tracheal epithelial (NHBE) cells, but not in normal human small airway epithelial cells (SAECs). (A) SAEC (left panels) and NHBE cells (right panels) were exposed to the indicated concentrations of SM, and cytosolic extracts were prepared after 16 h and subjected to quantitative fluorometric caspase-9 activity assays with Ac-LEHD-AMC (for caspase-9) as a substrate. Data are presented as the mean ± standard deviation of three replicates of a representative experiment; essentially, the same results were obtained in three independent experiments. (B) Cytosolic extracts that were prepared after 16 h in (A) were also subjected to immunoblot analysis, using antibodies specific for caspase-3 (upper panel) and capsase-9 (lower panel). The positions of the immunoreactive proteins are indicated.

NHBE cells exhibited a proteolytic processing of caspase-3 at a lower SM concentration (100 μM; Fig. 2B). When the immunoblot was stripped of antibodies and reprobed with antibodies to the initiator caspase-9, only a partial processing of procaspase-9, shown by the appearance of a larger cleaved form (p35), was noted in the SAEC. In contrast, NHBE cells exhibited a complete processing of procaspase-9 into its active form (p25), although the partially processed

p35 fragment was also evident and increased markedly after SM exposure. These results indicate that in large airway NHBE cells, SM-induced apoptosis occurs through both a DR-caspase-8 mediated pathway as well as a mitochondrial caspase-9 pathway, which results in an amplified response.

DISCUSSION

The present study demonstrates that both large (NHBE cells) and small (SAEC) airway epithelial cells undergo apoptotic signaling and death due to SM exposure *in vitro*, but distinct responses can be elicited. NHBE cells exhibit higher caspase-3 and -8 activities compared to SAEC, possibly due to an amplification loop downstream involving caspase-9 activation as a result of cross-talk between the DR and the mitochondrial pathways. The reason for these differences is unknown, but it may be due to different intrinsic properties that characterize the response of these cells to toxic stimuli.

In summary, the results of this study suggest that SM exposure induces a DR pathway of apoptosis in airway epithelial cells. The airway epithelium is a crucial structural and functional interface between the lungs and the environment. Upon SM entry into the respiratory tract, it has to first interact with airway epithelial cells, which respond by undergoing an apoptotic response, as evidenced by activation of a caspase cascade. Pulmonary A549 cells were also shown to exhibit an increase in DNA breaks, as detected by TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) staining and caspase-mediated PARP cleavage in response to SM exposure (Steinritz et al., 2007). Consistent with our results, SM-induced apoptosis of another human large airway bronchial/tracheal epithelial cell line, 16HBE, was correlated with increased p53 expression, Bax activation, decreased Bcl2 expression, opening of the mitochondrial permeability transition pore, release of cytochrome C, and caspase-2, -3, -8, -9 and -13 activation, thus indicating a mitochondrial pathway of apoptosis (Sourdeval et al., 2006). Inhibition of mitochondrial permeability transition protected the large airway epithelial cells against SM-induced apoptosis.

Based on our current *in vitro* results, both the large and small airways appear to be involved in SM toxicity with distinct patterns of responses. One type, in the large airways, induces an amplified apoptotic response *via* activation of mitochondrial pathway-associated caspase-9, probably downstream of caspase-8 and -3 activation. The other type, in the smaller airways, induces a DR-caspase-8 mediated pathway. Increased sensitivity of the NHBE cells to SM, relative to the SAEC, may be attributed to an amplification loop in bronchial/tracheal cells involving caspase-9 activation, probably resulting from cross-talk between the DR and the mitochondrial pathways. Cross-talk between the DR and the mitochondrial pathways has been shown to be mediated by Bid, a proapoptotic "BH3-only" member of the Bcl2 family. Bid is

cleaved and activated by caspase-8 in response to DR activation; the p15 form of truncated Bid (tBid) translocates to mitochondria and induces cytochrome C release, leading to the activation of downstream caspase-9 and apoptosis (Yin, 2000; Gross et al., 1999; Kim et al., 2000). Additional *in vitro* and *in vivo* studies are needed to validate the results of this study and to further define possible differences that may exist between upper and lower airway epithelial cells in terms of susceptibility and patterns of response to SM and to therapeutic intervention.

CONCLUSIONS

SM, which is known to cause respiratory tract injury, induces apoptosis in both NHBE cells (upper respiratory tract model) and SAECs (deep lung model). SM-induced apoptosis in NHBE cells and SAECs is demonstrated by the activation of the initiator caspase-8, a marker of the extrinsic Fas (DR) pathway, and caspase-3, the executioner caspase mediating the final steps of the apoptosis cascade (Fig. 1). Another initiator, caspase-9, a marker of the intrinsic mitochondrial pathway, is activated due to SM exposure in NHBE cells, but not in SAEC, suggesting that SM-induced apoptosis in SAEC does not involve caspase-9 (Fig. 2). Taken together, our results suggest that in the respiratory tract basal epithelial cells, SM causes apoptosis predominantly by a caspase-8-mediated pathway, which may serve as a target for the therapeutic intervention of SM inhalation injury.

ACKNOWLEDGMENTS

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